INHIBITION OF MITOCHONDRIAL MALATE DEHYDROGENASE BY 2-THENOYLTRIFLUOROACETONE

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1. Introduction

2-Thenoyltrifluoroacetone (TTFA) is a lipophylic iron chelating agent [1] which was regarded as a specific inhibitor of the mitochondrial succinate dehydrogenase, either in membranal [2] or soluble [3] forms. Boyer et al. [4] reported that it is also a mild uncoupler.

During the studies on the metabolism of the Krebs Cycle intermediates by intact rat liver or rat heart mitochondria, Gutman and Kearney [5] noted that oxidation of pyruvate plus malate was inhibited by TTFA. Malonate had hardly an effect on the oxygen uptake. This was taken as an indication that TTFA perturbed the oxidation of NADH by these mitochondria. As TTFA does not inhibit the oxidation of NADH by submitochondrial particles it was suspected that the inhibition is at one of the NAD reducing enzymes of the Krebs Cycle.

This assumption was tested by using rat-heart mitochondria which are practically devoided of glutamate dehydrogenase [6]. These mitochondria, in presence of malate, glutamate and arsenite, oxidise only the malate; the oxaloacetate formed is transaminated to asparate by the glutamate-oxaloacetate aminotransferase and the α keto-glutarate oxidation is inhibited by arsenite. In such a system TTFA inhibited the respiration while malonate had no effect at all [5]. Consequently it was concluded that in this case the malate dehydrogenase is the target for the TTFA inhibition.

The present communication documents that the mitochondrial malate dehydrogenase is indeed inhibited by TTFA, and evaluates the role of the malate on the observed level of the inhibition at finite substrate concentrations.

2. Materials and methods

Mitochondrial pig heart malic dehydrogenase was a Worthington preparation. (412 μmol NADH/min, mg 30°). TTFA was a Fluka AG. Buchs SG. product, recristallized and dissolved in ethanol. All the reactions were carried in 50 mM Tris–acetate pH 8.0, 30°C. The enzyme concentration was determined at 280 nm, using the value $E^{1\%}_{cm} = 3.05$ A [7]. The enzyme was diluted in the reaction buffer containing 2 μm NAD and was used within 2 hr after the dilution [8].

Oxidation of malate by NAD was measured in Hitachi MPF2 spectrofluorimeter. The excitation wavelength was 340 nm, and emission was measured at 460 nm. TTFA quenches the fluorescence of NADH. Because of that calibration curves, measured with the given concentration of TTFA, were used to calculate the rate of NAD reduction.

3. Results

Figs. 1 and 2 are primary plots relating the rate of NAD reduction to the malate and NAD concentration, measured in the absence (fig. 1) or presence (fig. 2) of TTFA. Each line in this figure was measured with a constant malate concentration ranging from 0.67 mM (0.75 K_m) up to 4 mM (5 K_m). ($K_{m\,(malate)}$ = 0.8 mM [9]).

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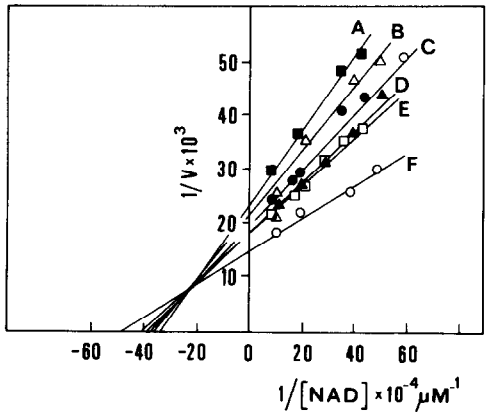

Fig. 1. Double reciprocal plots for the activity of malate dehydrogenase in presence of variable malte and NAD concentrations. Each line represents a set of measurements carried with the NAD concentrations as indicated on the abcissa and with constant malate concentrations. The malate concentration was A, 0.68 mM; B, 0.8 mM; C, 1.3 mM; D, 4 mM. The reactions was followed fluorimetrically in 50 mM Tris–acetate pH 8.0 at 30°C with 496 μg malate dehydrogenase/3 ml.

Figs. 3 and 4 are the secondary plots, where the $1/V_{max}$ values obtained from the primary plots are drawn with respect to the malate (fig. 3) or the NAD (fig. 4) concentration. The inhibition at infinite

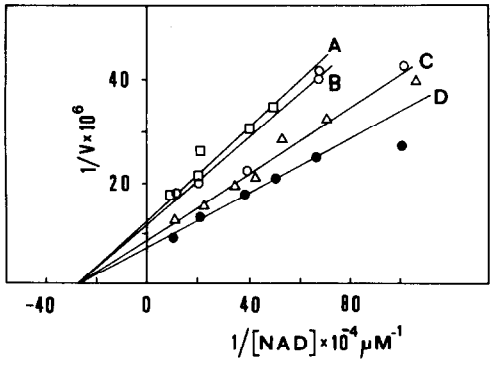

Fig. 2. Double reciprocal plots for the activity of malate dehydrogenase in the presence of 15 μM thenoyltrifluoroacetone. Each line represents a set of measurements carried with the NAD concentration indicated on the abcissa and with constant malate concentration. The malate concentration were A, 0.67 mM; B, 0.8 mM; C, 1 mM; D, 1.3 mM; E, 2 mM; F, 4 mM. The experimental conditions are as in fig. 1.

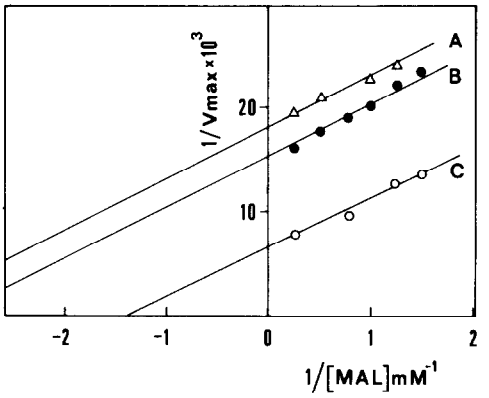

Fig. 3. Secondary plot for the effect of thenoyltrifluoroacetone on malate dehydrogenase activity. Each point represents the $1/V_{max}$ (NAD) measured with constant malate concentration. The data was extracted from figs. 1 and 2, and other experiments. A, 30 μm TTFA; B, 15 μm TTFA; C, no TTFA.

substrate concentration is non competitive with respect to NAD while with respect to malate is uncompetitive.

Fig. 5 relates the slopes of the lines in figs. 1 and 2 to malate concentration. At infinite malate concentration the slopes are identical but at diminishing malate concentration the slopes measured in presence of TTFA increase more steeply than in its absence. This

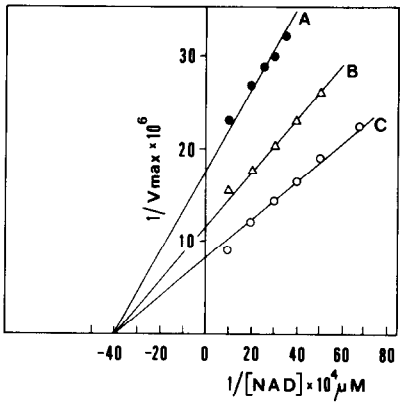

Fig. 4. Secondary plots for the effect of thenoyltrifluoroacetone on malate dehydrogenase. Each point represents $1/V_{max}$ (malate) measured with constant NAD concentration, data extracted from figs. 1 and 2, and other experiments. A, 30 μm TTFA, (K_i = 25 μM); B, 15 μM TTFA (K_i = 42.5 μM); C, no TTFA.

means that the inhibitor increases the dependence of the catalytic rate on the malate concentration; lowering the malate concentration will slow the rate in presence of TTFA, more than its effect in the absence of the inhibitor.

4. Discussion

TTFA has been considered as a specific inhibitor of the succinate dehydrogenase [1–3]. As documented here it inhibits malate dehydrogenase at the μM range, comparable to the concentrations which inhibit succinoxidase in mitochondrial or submitochondrial preparations.

The mechanism of the inhibition of malate dehydrogenase by TTFA is too complex for a straightforward interpretation. This is because the malate dehydrogenase binds its two substrates in a compulsory order [8,10], and the two subunits of the enzyme interact with each other during the catalytic cycle [10].

The detailed mechanism of inhibition is currently under investigation. In the meantime, we wish to point out that TTFA increases the dependence of the rate on the malate concentration (fig. 5). This is revealed as an increase in sensitivity at low malate concentrations, conditions which are encountered in intact mitochondria. In freeze clamped guinea pig liver the intramitochondrial malate concentration is 0.3–0.7 mM [11] while in rat heart mitochondria, the concentration is 0.3 and 0.38 mM for state 3 and state 4 respectively [12]. These levels are comparable or below the K_m = 0.8 mM [9]. This implies that studies in which TTFA was used to inhibit mitochondrial reactions should be reevaluated. A case in point might be the observations of Streichman and Avi-Dor [13] who noted that the reduction of NAD(P) by succinate in state 4 rat-liver mitochondria was more sensitive to TTFA than the oxidation of succinate. The studies of Hoberman and Pronsky [14], on the hydrogen pathway from succinate to acetoacetate in rat liver mitochondria, demonstrated the existance of an energy independent pathway operating via the fumarase, malate dehydrogenase system leading to reduction of NAD. Considering the possibility that some of the reduction of NAD(P), observed by Streichman and Avi-Dor, was carried by the pathway described by Hoberman and Pransky, then the extra sensitivity to TTFA [13] might be attributed to the inhibition of malate dehydrogenase.

Finally, the low malate concentration in the mitochondria tends to augment this inhibitory effect of TTFA. This might render the contribution of the fumarase–malate dehydrogenase system to be well distinct at low inhibitor concentration.

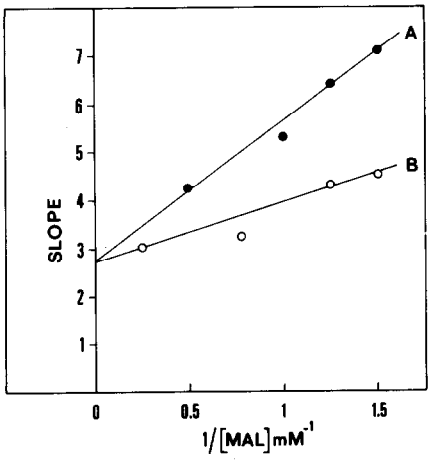

Fig. 5. The effect of malate on the slopes of the primary double reciprocal plots in the presence or the absence of thenoyltrifluoroacetone. Data extracted from figs. 1 and 2. A, 15 μM TTFA; B, no TTFA.

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